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Vannella pentlandii n. sp., (Amoebozoa, Discosea, Vannellida) a small, cyst-forming soil amoeba

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ABSTRACT

We describe a new species of cyst-producing soil amoeba *Vannella pentlandii* from course pasture in the Pentland Hills, Scotland. Analysis of the 18S rDNA gene reveals that it belongs to the sub-group within the genus, presently composed of *V. placida, V. epipetala* and *V. fimicola* (the PEF group). This group share features such as longitudinal folds/ridges on the lamella (the anterior hyaline region of the trophozoite), stubby floating forms and cyst production. While each PEF species contain cyst producing strains, not all strains within these species do so. *V. fimicola* produces cysts on stalks leading to its former classification as a slime mould, however no such stalks were evident in the *V. pentlandii*, instead groups of cysts become piled on top of each other forming clumps. The encysting amoebae crawl toward each other, pushing some off the surface to form these mounds. The *V. pentlandii* trophozoites are of typical size for the genus but the cysts at 6.9 μm in diameter, are the smallest so far described in genus *Vannella*. Other cyst producing species are found in various branches within the *Vannella* phylogenetic tree, probably meaning that this ability was ancestral but lost in many branches (particularly in marine species), and perhaps re-gained in others.

Keywords: *Vannella*; Cyst forming; Amoebozoa; Phylogenetics; Amoeba

This paper is dedicated to the memory of Dr Conrad A. King, a friend and mentor.

1. Introduction

The genus *Vannella* was created by Bovee in 1965 to include flattened, often fan-shaped amoebae with a large hyaline leading edge while in active locomotion (Bovee, 1965). *Vannella* was differentiated from *Platyamoeba* as the later did not possess glycostyles visible under E.M. but instead had a "fuzzy" glycocalyx which may be arranged in a hexagonal pattern (Page, 1988). Electron microscopy of the early branching Vannellid, *Paravannella minima* (Kudryavtsev, 2014) indicates that the ancestral state appears to bear long pentagonal glycostyles and simple, longer filaments that have subsequently been lost in some groups. Until the year 2000, another recognised distinction between *Vannella* and *Platyamoeba* was that *Vannella* lacked the cyst stage that *Platyamoeba* may have, however one species, *V. persistens,* was discovered which had a cyst stage (Smirnov & Brown, 2000). Later genetic studies made it clear that the distinction between *Vannella* and *Platyamoeba* is not a natural one, and all "*Platyamoeba*" members were transferred to genus *Vannella* (Sims et al., 2002; Dyková et al., 2005; Smirnov et al., 2007). A number of workers had studied various aspects of the amoeba *Hyalodiscus simplex* (Wohlfarth-Bottermann, 1960) particularly aspects of its locomotion (Hülsmann & Haberey, 1973) and it too was reclassified as belonging to the genus *Vannella* (Bovee, 1965), although this seems to have been initially resisted (Hausmann, 1975).

Within the genus *Vannella* as it is currently viewed, only a few species are reported to be capable of producing a cyst. In addition to *V. persistens*, these include *V. schaefferi* (Page, 1988), *V. placida* (Page, 1968), *V. contorta* (Moran et al., 2007), *V. danica* (Smirnov et al., 2002) and a *Vannella* from Iran (Lasjerdi et al., 2011). In other species the cyst forming status is less certain. *V. epipetala*, for example is reported to produce a cyst-like structure after incubation above 30^{o}C, no amoebae could be recovered from these (Amaral-Zettler et al., 2006). Images of cysts from a second *V. epipetala* isolate from Jamaica have also been published (Todd et al., 2015). Cysts were seen in early cultures of *V. ebro* but their development could not be stimulated in later cultures (Smirnov, 2001). The occurrence of cysts in the SIA strain of *V. placida* was described as "when present" indicating their scarcity (Sims et al., 2002) and two strains identified from incomplete 18S gene sequences as being *V. placida* have been isolated from bat guano but neither produces cysts (Mulec et al., 2016).

Although there is presently no indication that *Vannella* itself is pathogenic, like other free living amoebae (FLA) it can facilitate the growth of bacteria (Loret et al.., 2008; Schulz et al.., 2015) including *Legionella* (Kuroki et al., 1998) and other organisms (Scheid, 2007), some of which are human pathogens. This makes the study of the cyst stage of *Vannella* important since pathogens are likely to be protected in these cysts as is known to be the case for other FLA. Again like many other FLA, *Vannella* are commonly found in the human water supply (Thomas et al., 2008; Poitelon et al., 2009), in our domestic appliances (Rivera et al., 1993) and even on our crops (Chavatte et al., 2016).

Here we describe a new species of *Vannella* which produces abundant cysts in an unusual cooperative manner in which amoebae aggregate prior to encysting forming hemispherical mounds on agar surfaces.

2. Methods and Materials

2.1 Isolation and culture of amoebae

The root systems of individual *Capsella bursa pastoris* plants were collected and placed in sterile plastic bags. Amoebae were washed off the roots by vortexing in ice cold, sterile Neff's saline (Page, 1988) and spread on Neff's saline 2% agar plates overnight. Strips of these plates were then cut and inverted on *E. coli* spread 2% agar plates containing 0.01% yeast extract and 0.025% malt extract in Neff's saline (YME plates) and incubated at room temperature. These plate were monitored daily using an inverted microscope and blocks of agar supporting amoebae were excised and inverted on fresh *E. coli* spread YME plates. This process was repeated in order to isolate particular clones. Cultures were found to be more productive if they were kept on *E. coli* spread plates rather than the more usual overlay with Neff's saline as the bacteria tended to overgrow the amoebae. This amoeba grew well at 30ºC but failed to grow at 37ºC.

2.2 Cryopreservation

Amoebae and cysts were taken up in Neff's saline with 10% DMSO and placed overnight at -20ºC, then transferred to -80ºC for long term storage. Live cells were recovered by settling the thawed vial content after replacing the medium with Neff's saline by centrifugation, onto non-nutrient agar for an hour after which the media was removed and excised blocks inverted upon *E. coli* spread plates containing 2% agar, 0.01% yeast extract and 0.025% malt extract in Neff's saline. This method was found to return viable cultures after at least two months of frozen storage.

2.3 Light microscopy

Light microscopic observations were made on living amoebae using an inverted Leica DMIRB microscope equipped with Hoffman modulation contrast optics and a Canon EOS1100D camera controlled by laptop computer with Canon software. Time lapse (a frame every 30 seconds) videos were recorded of encysting amoebae.

2.4 Fluorescence microscopy

Cysts were harvested from agar plates and washed twice in Neff's saline, stained with 10 µg/ml Hoechst 33342 for 20 minutes, and washed once more. Cysts were then placed on a coverslip and viewed with a Nikon A1R microscope excitation at 350 nm, emission at 461 nm. Images were processed using Image analysis Software (Imaris).

2.5 Genomic DNA purification and PCR

Genomic DNA was isolated and purified as previously described (Lorenzo-Morales et al., 2005). Briefly, amoebae were lysed and treated with proteinase K at 60ºC for 2 hours followed by phenol-chloroform extraction and isopropanol precipitation. The resulting DNA was quantified spectrophotometrically. 100ng of genomic DNA was used per PCR reaction using HotStarTaq polymerase (Qiagen). The eukaryotic 18S several primer pairs as described (Corsaro and Venditti, 2010). CAT1 (5′-CAT GCA TGT CTA AGT ATA AGC-3′) with GSPr (5′-TTC AC <G/A> GTA AAC <G/A> ATC TGG GC-3′), or 1137R (5′-GTG CCC TTC CGT TCA AT-3′), and 892cF (5′-GTC AGA GGT GAA ATT CTT GG-3′) with Br (5′-GAT CCT TCT GCA GGT TCA C-3′). A fragment of the COI gene was amplified using forward primer LCO1490f 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3' and reverse primer HCO2198r 5' TAA ACT TCA GGG TGA CCA AAA 3' (Folmer et al., 1994; Nassonova et al., 2010). PCR conditions were 15 min at 94°C, followed by 30 cycles of 94°C for 30 s, 55°C 30 s, 72°C

2 min, with final extension of 72°C 5 min. PCR products were purified with a QIAquick PCR purification kit (Qiagen) and sequenced by Edinburgh Genomics using the same set of primers.

2.6 Phylogenetic analysis

Sequences were obtained for a wide range of *Vannella* strains from Genbank and compiled together with the new sequences using "Seaview", version 4 (Gouy et al., 2010). "BioEdit" (Hall, 1999) was used to trim sequences and to determine levels of homology between sequences. Maximum likelihood phylogenetic trees were obtained using PhyML software (Guindon and Gascuel 2003) using the GTR model. The non-parametric analysis was performed with 1000 bootstrap pseudo-replicates, using representative sequences from other amoebozoans (*Acanthamoeba*, *Amoeba* and *Chaos*) as the outgroup.

3. Results

Diagnosis. The locomotive form often semi-circular and occasionally with a pronounced tail. The flattened, leading lamella is devoid of organelles which are limited to a mound-shaped body toward the rear of the cell. There is a usually a single contractile vacuole visible with a periodicity of 127 seconds (S.E. = 9.2) at 24ºC in Neff's saline, in accordance with other freshwater/soil members of the genus under similar physical circumstances (Ariza et al., 1989). The length of the locomotive form varies between 22 - 40 μm (average 28 μm). The breadth range is 12 - 27 μm (average 20 μm), making the L/B ratio about 1.4. The predominantly single nucleus is 3 - 4 μm in diameter and vesicular, with a central nucleolus in the trophozoites while the nucleus is 2 μm in diameter in the cyst. Faecal pellets have not been observed adhering to the amoebae as they have for example in strains of *Vannellla simplex* (Smirnov et al., 2002*)*. The floating form has multiple, short-stubby finger-like projections, tending not to exceed the diameter of the cell mass. The cysts range between 6 and 10 μm in diameter (average 6.9 μm), and are usually present in large mound-shaped aggregates on agar plates.
Type location: Soil from the roots of a small, apparently healthy "Shepherd's purse" (*Capsella bursa pastoris*) plant from rough grazing (primarily sheep) in the Pentland hills, near Silverburn, Midlothian, Scotland. Grid reference 55.832636, -3.261551, at an elevation of 281m.
Type material. The live strain is held at the Culture Collection of Algae and Protozoa at Oban, Argyle, Scotland, with the accession code CCAP 1589/23. 18S rDNA gene and the mitochondrial COI gene sequences obtained from the type strain were deposited with GenBank accession numbers KY344796 and KY344797 respectively.
Etymology. Named after the Pentland hills from where this species was first isolated.
Differential diagnosis. The newly described *V. pentlandii* clearly differs from all other *Vannella* for which 18S rDNA gene sequence information is available, but is most similar to *V. placida* (first described as *Rugipes placidus*) (Page 1968). Morphologically too it is most similar to *V. placida* but there are important differences. *V. pentlandii* is proportionally narrower (Table 1). There is often a pronounced "tail" as there is in other species such as *V. croatica* (Smirnov et al., 2016; Supp figs). In locomotion *V. pentlandii* only rarely show the longitudinal ridges possessed by *V. placida* (Sims et al., 2002). The cysts described for the original *V. placida* strain, produced spherical and smooth with the nucleus eccentrically positioned. The cyst nuclei of *V. pentlandii* were usually not visible under conventional light microscopy but fluorescence microscopy (Fig. 1e) revealed that the Hoesch stained nuclei were central, unlike *V. placida,* and to be around 2 μm in diameter. Phase contrast images of *V. pentlandii* cysts (Fig 1 f) did not show the "broad white ring" inside the cyst wall described in

V. placida (Page, 1968). *V. placida* cysts have a double wall and are 7 - 10.5 µm in diameter whereas those of *V. pentlandii* appear to have a single wall and are slightly less at 6.9 µm.

The locomotory morphology of amoebae on glass, plastic and agar was compared since some *Vannella* species do not adhere to glass well (Page, 1980), *V. nucleolitateralis* amoebae were found to be more elongate and linguiform on plastic than those on glass (Anderson et al., 2003) and *V. ebro* differed when locomoting on plastic compared to glass (Smirnov, 2001). We found that *V. pentlandii* adhered loosely to both glass and agar but more firmly to tissue culture plastic, however there was no significant difference in the length or breadth measurements in the amoebae while locomoting on these surfaces (Fig. 2). This is in accordance with the finding that *Vannella* can polarise and locomote on the air-water interface (Preston 2003) where strong adhesions cannot be made.

The sequence analysis of the SSUrDNA PCR product of *V. pentlandii* confirmed that this strain belongs to the genus *Vannella*. A phylogenetic analysis was conducted comparing our new sequence data to a range on other *Vannella* sequences. The tree agrees with several other published trees (Dyková et al., 2005, Nassonova et al., 2010, Kudryavtsev 2014, Smirnov et al., 2016) in that the genus *Vannella* consists of at least 10 main groups, and that the marine members form a distinct branch (Fig 4). There are about 5 marine groups and 5 freshwater/soil groups but these of course differ in their distinction from other groups and our study did not include all the sequences available. This analysis also reveals that *V. pentlandii* belongs to a group currently consisting of *V. placida*, *V. epipetala* and *V. fimicola*, (the PEF group) all of which tend to various extents to produce cysts. Morphologically, the PEF group are also united by the possession of a stumpy floating form (Page, 1968; Amaral-Zettler et al*.,* 2006). The production of longitudinal folds on the lamellae of *V. pentlandii* (Fig 1b) is shared by at least two others of the PEF group, GERL41 (Dykova et al., 2010) and *V. placida* (Sims et al., 2002), however these folds or ridges are not a frequent feature of *V. pentlandii*. These longitudinal folds may constitute a useful diagnostic marker for the DEF groups within freshwater and soil *Vannella*, but it has yet to be described in *V. epipetala* or in *V. fimicola*. Longitudinal folds have also been described in two marine species, *V. ebro* (Smirnov, 2001), and *V. weinsteini* (Sawyer 1975).

We have also amplified the mitochondrial COI gene from *V. pentlandii* and built a phylogenetic tree of freshwater and soil *Vannella* using the available GenBank data. The COI tree broadly agrees with the 18S rDNA gene tree but is much less detailed because of the current relative dearth of Vannellid COI gene sequences. The tree naturally agrees with that of the source of the majority of the data in our tree (Nassonova 2010).

The observation that *V. fimicola* produces stalked cysts encouraged us to investigate the possibility of stalk formation in *V. pentlandii*. Although no stalks were observed directly when the amoebae formed cysts on *E. coli* spread agar, we did find that the cysts formed in groups and that many cysts within these clumps were in a different plane of focus as the agar surface indicating the possibility for the existence of stalks. In order to see "from the side", we allowed the cysts to form on glass wool (Fig. 3). This allowed us to observe that amoebae grouped together in approximately spherical aggregate to form cysts and that in this way cysts formed that were stuck together by an unknown material that was produced on the surface of the forming cysts. We were able to observe an amoeba climbing on top of a cyst and then beginning to encyst itself giving a clue as to how these clumps form in three dimensions (Fig. 3b). However, no actual stalks were observed, even after the glass wool dried out. Time-lapse video microscopy (see supplementary data) was used to study the formation of the cyst aggregates when the amoebae are placed on bacterial lawns. Cells were observed to wander around and stick loosely to each other as they collided, and these small groups then attracted other

amoebae. Some trophozoites were seen to interact with many other groups before finally settling for one. The amoebae at the periphery of the gathering groups seemed to push others up and off the agar surface resulting in the hemi-spheroidal mass of encysting cells.

4. Discussion

Vannella is an abundant genus, found in a large variety of environments, in all regions of the world. It is particularly numerous and ubiquitous marine environments from the surface waters to abyssal planes, hydrothermal chimneys (Sauvadet et al., 2010) and present even in anoxic marine sediments (Smirnov & Fenchel, 1996). Phylogenetic analyses have highlighted a degree of separation of these marine species into groups distinct from freshwater/soil groups (Sims et al., 2002; Dyková et al., 2005; Smirnov et al., 2007). Our analysis also demonstrates this and also that the marine species tend not to produce cysts as frequently as terrestrial species. Currently, *V. contorta* is the only known cyst producing fully marine Vannellid (Moran et al., 2007), and it is possible that this is a response to its extreme salinity changes and low temperatures found on the pack-ice surface. The position of *V. danica* among freshwater members is curious since it was isolated from brackish environment, but it is probable that it is related to freshwater strains but that it has since adapted to the higher salinity of the brackish water from which it was isolated. One other "marine" isolate (ECH30) also groups with freshwater/soil members but this was from inshore location and so may be of terrestrial origin too. Perhaps the scarcity of cyst forming Vannellids and other marine amoebae (Page 1983) reflect the relative constant conditions in the seas and oceans?

On land the genus has been found in extreme conditions (Mulec et al., 2016), from hot springs (Niyyati & Latifi, 2017), to deserts (Rodríguez-Zaragoza & García, 1997; Mayzlish-Gati & Steinberger, 2007) and tropical forest (Todd et al., 2015). Part of their ability to endure these extreme and changeable conditions is likely to be due to the cysts as is the case for other amoebae and other protists, yet there is very little information about the cyst stage of Vannellids. The presence of cyst producing species in many separate branches of the *Vannella* phylogenetic tree (largely excluding the marine groups) and on many other groups of Amoebozoa probably means that cyst production was an ancestral property that many species have now lost. It is also probable that some lineages have re-established a cyst forming ability after having once lost it. The fact that *V. pentlandii* cysts stain with calcofluor probably means that in common with other FLA (for example *Naegleria*, *Acanthamoeba*) these cysts contain cellulose or a cellulose-like polymer (Tomlinson & Jones, 1967).

Our sequence analyses of 18S rDNA gene sequence concludes that *V. pentlandii* is a member of soil/freshwater Vannellids with a tendency to produce cysts (the PEF group). Another member of this group is *Protosteliopsis fimicola* now known to be a Vannellid (Shadwick et al., 2009) and not a protostelid as originally suggested and it has been renamed "*Vannella fimicola*" (Van Wichelen et al., 2016; Cavalier-Smith et al., 2016). The species name *fimicola* means "living on dung" from where it was fist isolated (Olive, 1962) and so remains suitable. It seems likely that *V. fimicola* was confused for a simple slime mould because the stalked morphology of the cyst strongly resembles a fruiting body. The stalk is produced by an individual amoeba as it rises making the stalk (8 - 36.5 µm in length) and then encysting at the highest point off the substrate. The original description (Olive, 1962) used the word "spore" instead of "cyst" but this is unwelcome since "spore" implies haploid formation in many biological contexts, especially in botany. At present it is not clear if other *Vannella* species produce a stalk of any kind, however *V. pentlandii* certainly produces groups of cysts like *V. fimicola*, and some of these "pile-up" on each other to form rounded clumps. It is also unclear

if the *V. pentlandii* cysts can make stalk-like structures given suitable conditions or if material similar to that comprising *V. fimicola* stalks is responsible for aggregating *V. pentlandii* cysts. The fact that these stalks dissolve rapidly in water makes this awkward to study, but clearly a direct comparative study between the species within the immediate group is desirable. The formation of the aggregates of encysting amoebae is interesting but the advantage that this brings to this species is not immediately obvious. Perhaps this aggregation has some sexual or parasexual purpose but we have no data to support this hypothesis. It is also possible that aggregates increase survival by more efficient dispersion, as is presumed to be the case for the stalked cyst arrangement of *V. fimicola*.

The SSUrDNA gene has been used to determine the taxonomic relations of many amoebal groups including *Vannella* (Smirnov, 2007; Kudryavtsev, 2014) but the situation in the genus *Vannella* is particularly problematic as there is micro heterogeneity within the gene in this genus. It is suggested that other genes, such as the mitochondrial COI gene may be more useful (Nassonova et al., 2010). In the absence of a well-defined species concept for amoebae it has been suggested that we might consider the COI phylotypes as molecular operational taxonomic unit (Nassonova et al., 2010) until it can be agreed how we can define a species within the amoebae. However, there are relatively few COI sequences currently available for *Vannella*, and so the taxonomic information from the *V. pentlandii* COI gene is presently limited.

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Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version

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Species	Length / Width (µm)	Known habitats	Floating form	Cyst diameter (µm)	Reference
V. danica	25—50 25—50	Brackish water (17ppt)	1-9 pointed, tapering hyaline pseudopodia	16–26 single walled	Smirnov et al.., 2002
V. epipetala	20.2–28.6 23.4–37.1	Leaf surface & Freshwater	Body contracted only very short protrusions	Cysts not characterised but about 20	Amalar-Zettler et al.., 2006 Todd et al.., 2015
V. persistens	23–35 23–40	Soil & Freshwater	1–8 tapering pseudopodia of different lengths	13–16 double walled	Smirnov & Brown, 2002
V. placida	15-35 9-66	Freshwater & Soil	Short finger-like pseudopods	7-10.5 double walled	Page, 1968; 1988 Sims et al.., 2002
V. contorta	12-29 12-23	Marine pack ice Antarctic	Several blunt short pseudopodia	8-18 Thin fibrous walls	Moran et al.., 2007
V. pseudovannella	8.5-25.5 6.8-25.5	Hypersaline lake	Long, tapered pseudopodia	About 10 but appear to be dehydrated amoebae.	Hauer et al.., 2001
V. ebro	25–40 35–60	Cyanobacterial mats 70-80 ppt	4-8 long, tapering blunt tipped pseudopodia	Cysts only in fresh isolate	Smirnov, 2001
V. fimicola	c22 c22	Soil/dung	undescribed	Cysts on stalks 10-16	Olive 1962 Olive 1967
V. pentlandii	14-30 11-20	Soil	Short finger-like pseudopods	6.9 single walled	This study

Table 1. Characterization of the known and possible cyst-forming *Vannella* species.

Figure Legends

Figure 1.
a, b & d) Locomotory morphology of the trophozoite in Neff's saline, on tissue culture plastic. b) A form showing longitudinal folds/ridges on the lamellae during locomotion. c) Floating form. e) Confocal microscopy Hoescht 33342 stain (blue) showing centrally located nucleus in two cysts. f) Corresponding phase contrast of the cysts. Bars are 5μm.

Figure 2.
Length (L) and breadth (B) measurement of *V. pentlandii* trophozoites locomoting on tissue culture plastic, glass and 2% agar surfaces in Neff's saline. Error bars are SD. There is no significant difference (two tailed t-test) between the lengths nor the breadth measurements of trophozoites on the three different substrates. There is a significant difference between the lengths and breadth except for those amoebae measured on agar substrates.

Figure 3.
a) Amoebae gathering together on *E. coli* spread plate to form cyst aggregate. b) Cyst formation on glass wool to show "from the side" views. Amoebae in Neff's saline slowly starved and dehydrated initiating cyst formation. b) Cysts in a spheroidal clump. c) An amoeba (arrow) climbed on top of a preformed cyst and rounded up, presumably as a prelude to encystment.

Figure 4.
A PhyML phylogenetic tree (GTR model) of members of the genus *Vannella* based on 18S rDNA gene. Branch support values at each node indicated as percentages. The GenBank accession code of each sequence is followed by the species names, then strain name (where available). The tree has been rooted using an number of other amoebozoans as the outgroup. Those species that are known to produce cysts are marked with black closed circles. *Vannella* known to produce longitudinal folds on the lamella (Fig 1b) are marked with open circles. Bootstrap analysis was performed using 1000 replicates. The scale bar shows the evolutionary distance for the nucleotide substitutions per site.

Figure 5.
A PhyML phylogenetic tree (GTR model) of freshwater and soil *Vannella* based on the mitochondrial COI gene (Nassonova et al.., 2010). The tree was rooted with a *Cochliopodium* COI gene (GQ354207). Bootstrap analysis analysis was performed using 100 pseudo-replicates. The scale bar shows the evolutionary distance for the nucleotide substitutions per site.

Supplementary data.
A time-lapse video showing the aggregation of *V. pentlandii* trophozoites was recorded. The 8.5 hr recording follows the formation of new aggregates. These condensed and threw up encysting cells out of the plane of focus where they formed cysts.